



Intentional summer flooding of an *Avicennia germinans* mangrove forest has a more direct effect on ammonia-oxidizing Betaproteobacteria than on Thaumarchaea

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Abstract

Aims Rotational Impoundment Management (RIM) involves summer inundation of impounded mangrove forests for mosquito management. The goal of this study was to investigate the impact of RIM on communities of aerobic ammonia-oxidizing microorganisms in *Avicennia germinans* dominated mangrove forest soils.

Methods Soil samples were collected annually in a managed and an adjacent, non-managed impoundment before and after the start of RIM at three elevation levels with their characteristic mangrove habitats, *i.e.*, dwarf (highest elevation), sparse and dense

(lowest elevation). The ammonia-oxidizing communities were studied by qPCR and amplicon analyses based on thaumarchaeal and betaproteobacterial *amoA* genes.

Results Temporal variations in copy numbers and assemblies of *amoA* gene amplicons were limited. Thaumarchaeal *amoA* genes increased in the dwarf and sparse habitat in the non-managed impoundment, and betaproteobacterial *amoA* genes increased in the dwarf habitat in the RIM impoundment. No copies of the *amoA* gene of Nitrospirota (comammox bacteria) were detected in either impoundment. Whereas there were no significant effects of RIM on the composition of thaumarchaeal communities, RIM affected the composition of betaproteobacterial *amoA* assemblies in all habitats in the RIM impoundment.

Conclusions Direct consequences of RIM were reflected in changes in the composition of assemblies of amplicon sequence variants (ASVs) of ammonia-oxidizing Betaproteobacteria in all mangrove habitats of the RIM impoundment. Significant temporal changes at higher elevations in the non-managed impoundment were likely due to groundwater exchange between the impoundments.

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Introduction

Mangrove forests, which are situated at the interface between terrestrial and marine ecosystems in tropical and subtropical areas, do not only offer a large number of ecosystem goods and services (Duke et al. 2014), but provide habitat for nuisance mosquitos (Rey et al. 2012b). To manage mosquitos in Florida, a number of mangrove forests along the Indian River Lagoon were impounded several decades ago (Rey et al. 2012b). In March 2009, Rotational Impoundment Management (RIM) was introduced in one of the mosquito control impoundments to control mosquitos and also improve the growth of mangroves (Rey et al. 2012a). In RIM, impoundments are flooded by pumping estuarine water into the impoundments during the summer mosquito production season. During months when mosquito populations are not being controlled by RIM, tidal exchange between the estuary and the impoundment occurs through culverts.

Mangrove vegetation in the impoundments is characterized by a gradient of tree heights and densities with taller and denser trees in areas that flood more frequently and to a greater water depth. At the other end of the height and density gradient are habitats that flood less frequently. Mangroves in those areas are shorter and density is lower (Verhoeven et al. 2014). Within 5 years of RIM implementation, the proportion of dense mangrove vegetation increased from 59% to 74% of the total area of the impoundment (Oostdijk et al. 2018). In contrast, the fraction of dense vegetation remained constant in an adjacent, non-managed impoundment. Vigorous plant growth in the dwarf habitat in the RIM impoundment may have caused an ammonium limitation for the aerobic ammonia-oxidizing microbial community, since the nitrogen-use efficiencies in mangroves are among the highest recorded for angiosperms (Reef et al. 2010). Ammonium was for example depleted in the root zone of the mangrove species *Kandelia obovata* (formerly *Kandelia candel*) (Alongi et al. 2005).

Simultaneously with the increase in vegetation in the RIM impoundment (Oostdijk et al. 2018), soil moisture content, total nitrogen, and potential denitrification activity (PDA) increased, while salinity decreased after the start of intentional summer flooding, but the changes only occurred in the dwarf habitat (Laanbroek et al. 2024). In the two other habitats (sparse and dense), total nitrogen and PDA

increased independent of summer flooding, whereas RIM did not affect soil moisture content and salinity. Labile organic nitrogen increased only in the RIM impoundment in all three habitats. Potential nitrifying activity (PNA) was generally not affected, except in the dwarf habitat.

The absence of a clear effect of RIM on PNA would suggest that the microorganisms responsible for aerobic nitrification were also not affected by intentional summer flooding. In a previous study, we showed that the presence or absence of tide overruled site-specific effects of mangrove species on microbial communities including those of aerobic ammonia-oxidizing Thaumarchaea and Betaproteobacteria (Marcos et al. 2018). Aerobic ammonia-oxidizing microorganisms are the first and often rate-limiting group of organisms in the process of aerobic nitrification, which is key in the global nitrogen cycle as it connects the oxidative and the reductive parts of the cycle. Changes in oxygen and ammonium availabilities that might be due to increased moisture contents and plant growth, respectively, as well as alterations in salinity and pH may lead to changes in the microbial community with a decrease in ammonia-oxidizing species by species that are more adapted to newly created conditions (Jung et al. 2021). Shifts in dominant ammonia-oxidizing Betaproteobacteria for example have been shown along a salinity and oxygen gradient in the Scheldt estuary (de Bie et al. 2001; Bollmann and Laanbroek 2002). In the present study, we investigated potential changes in the microbial community following RIM by analyzing *amoA* genes in soil samples collected before and after the implementation of RIM. Samples from the RIM impoundment were compared to samples collected in an adjacent, non-managed impoundment. As summer inundation is expected to affect the different elevations and corresponding habitats differently, the study was in each impoundment, replicated in the three distinctively different mangrove habitat zones, *i.e.*, dwarf, sparse, and dense. Results from this study will increase our understanding of potentially different responses of thaumarchaeal and betaproteobacterial ammonia-oxidizing microorganisms to environmental changes caused by adaptations in water management in mangrove ecosystems.

Methods

Study sites

The two study impoundments (#23 and #24) are located in the Indian River Lagoon, Florida, USA and have been described in detail (Laanbroek et al. 2024). Impoundment #23 was originally constructed in 1966 and was not open to tidal exchange until 1974 when its dike was breached (Rey and Kain 1991). Since then, tidal water had free entrance through the breach and two culverts (Feller et al. 2003). Impoundment #24 was constructed in 1970 and in 1985 and 1987 culverts were installed to enable management of tidal exchange between the impoundment and the Indian River (Whigham et al. 2009). In March 2009, rotational impoundment management (RIM) was introduced in impoundment #24 to improve mosquito control and plant growth. RIM consists of pumping Indian River Lagoon water into the impoundment during between March and September. In other months, the culverts are open and there is tidal exchange with the Indian River. The management of impoundment #23 remained the same throughout the sampling years. Impoundments #23 and #24 are hereafter referred to as the non-managed impoundment and the RIM impoundment.

Vegetation in both impoundments is dominated by black mangrove (*Avicennia germinans*) with scattered white mangrove (*Laguncularia racemosa*) in the interior and red mangrove (*Rhizophora mangle*) at the fringe adjacent to the perimeter ditch at the inner side of the dike (Laanbroek et al. 2024). In addition to the black mangroves, all three areas had a few other salt-tolerant species, especially saltwort (*Batis maritima*). In the RIM impoundment, there were areas (salt pans) that had no vegetation or scattered dwarf mangroves and *B. maritima* prior to the start of RIM in March 2009.

Collection and treatment of soil samples

Soil samples were collected in both impoundments in early March in 2008, 2009, 2013 and 2014 in three habitats. In each impoundment, 15 sample plots of 1 m² were established (5 in each habitat) and sampled each of the four years. No significant differences in soil texture were observed for the different sampling locations (Laanbroek et al. 2024).

Because of the time required to process the soil samples, the two impoundments were sampled at two- to three-day intervals. At each sampling location, three soil cores were collected from the surface in aluminum tubes (3.9 cm diameter and 10 cm long), which were sealed with rubber stoppers at both ends and transported at outside temperature within four hours to the Smithsonian Marine Station (SMS) at Fort Pierce located at 16 km distance from the sampling locations.

Immediately at arrival at SMS, the soil cores were prepared for further physicochemical and biogeochemical analyses (Laanbroek et al. 2024). One of the three cores collected at one sampling location was used for the determination of pore water characteristics, *i.e.*, salinity, pH, ammonium, nitrate, iron, calcium, magnesium, sodium, potassium, phosphate, and sulfate. The upper 5 cm of the remaining two cores was combined, mixed by hand, and divided into five subsamples of 20 g each for further analyses of total carbon and nitrogen, of moisture and dry bulk density, of 1 M KCL extractable ammonium, nitrate, and phosphate, of labile organic nitrogen, and of potential nitrifying and denitrifying activities, all as described in detail earlier (Laanbroek et al. 2024). A subsample of 5 g was freeze-dried at -20 °C for DNA-based microbial community analyses.

DNA isolation

Total genomic DNA was extracted from freeze-dried soil by homogenizing 0.5 g soil in 1 ml cetyltrimethylammonium bromide (CTAB) buffer, followed by two rounds of bead-beating at 5.0 m/s rotation for 1 min. Subsequently, samples were incubated at 37 °C for 30 min in the presence of 5 µL proteinase K (20 mg/ml) with regular intervals of 15 min vortexing to homogenize samples. Samples were then supplemented with 150 µL of 20% sodium dodecyl sulfate (SDS) solution and incubated at 65 °C for 1 h in a thermoblock. Every 15 min the samples were homogenized by vortexing. Samples were centrifuged at 10,000 g for 10 min, and 500 µL of the supernatant was collected and directly added to the lysis buffer of the Maxwell®16 (Promega, Fitchburg, Wisconsin USA) DNA tissue extraction kit. The automatic purification of genomic DNA was performed using the tissue DNA extraction program of the Maxwell kit.

Quantitative amoA-based PCR

To quantify thaumarchaeal and bacterial *amoA* genes a Rotor-Gene® Q thermocycler (QIAGEN, Hilden, Germany) was used with the following primer sets: Arch-*amoA*F (Francis et al. 2005) / Arch *amoA*_175Brev (Laanbroek et al. 2018), and *amoA*-1F (J) (Rotthauwe et al. 1997) / *amoA*_405Crev (Laanbroek et al. 2018). Reactions were performed in 20 µL volume containing 5 µL DNA solution, 1 µL of a 5 µM solution of each primer, 1 µL BSA and 10 µL of SYBR Green thereby using the Rotor-Gene SYBER Green PCR Kit (Qiagen, Hilden, Germany), specific for the Rotor-Gene 6000 (Qiagen, Hilden, Germany). The qPCR was conducted in 40 cycles with annealing temperatures of 50 °C for thaumarchaeal *amoA* and 55 °C for betaproteobacterial *amoA*. Clones of *E. coli* JM109 containing the thaumarchaeal or the bacterial *amoA* gene fragment were included as standard. All DNA samples and a negative control without DNA were analyzed in duplicates to obtain an accurate value for the *amoA* gene abundance in each sample.

Sequencing of *amoA* gene amplicons for ammonia-oxidizing Thaumarchaea and Betaproteobacteria

For sequencing with the Illumina MiSeq sequencing platform, the primer sets (1) Arch-*amoA*F / AOA_ *amoA*_175Brev, (2) *amoA*1F / AOB_ *amoA*_405Crev (Laanbroek et al. 2018) and (3) Ntsp-*amoA* 162F/ Ntsp-*amoA* 359R (Fowler et al. 2018) were used to amplify the *amoA* gene fragments of ammonia-oxidizing Thaumarchaea, Betaproteobacteria and comammox Nitrospirota, respectively. The PCR conditions for thaumarchaeal and betaproteobacterial *amoA* were those described by before (Laanbroek et al. 2018). One µl of genomic DNA was used as a template for each reaction mix. Triplicate reaction mixtures per sample were pooled together, purified with the Agarose Gel DNA purification kit (TaKaRa), and quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, USA). The bar-coded PCR products from all samples were normalized in equimolar amounts before sequencing. The amplicon library was prepared by adaptor ligation and PCR using the TruSeq nano DNA Library Prep Kit (Illumina, CatFC-121–4001)

according to the TruSeq nano protocol (Illumina, FC-121–4003). Paired-end MiSeq sequencing was carried out by Quebec Genome Center.

Sequencing data processing and identification of thaumarchaeal and betaproteobacterial *amoA* amplicon sequence variants (ASVs)

Adapter and primers were removed with Cutadapt v2020.11.1 (Martin 2011). To identify ASVs, forward and reverse reads were processed using dada2 as implemented in qiime2 v2019-08 (Callahan et al. 2016; Bolyen et al. 2019). Quality trimming, denoising, merging, and chimera detection were performed using the qiime2 v2020.11.1 plugin “qiime dada2 denoise-paired” with default settings except for “-p-trunc-len-f” and “-p-trunc-len-r” which were set at 130 and 155 nucleotides, respectively. For *amoA*-AOB sequences, the plugin classify-sklearn (Pedregosa et al. 2011) was used to classify the taxonomic lineage of the representative sequences of the resulting ASVs based on a custom bacterial *amoA* database, built using NCBI sequences (NCBI Coordinators 2015) and an in-house pipeline (Costa et al. 2022). For *amoA*-AOA data, the ASV representative nucleotide sequences were analyzed using BlastN against the GenBank nucleotide database (NCBI Coordinators 2015). The ASV tables were converted into tab separated values (tsv) format and exported using the BIOM package (McMurdie and Paulson 2020).

Statistical analyses

Statistical analyses of the annual inundation data, measurements of abiotic soil characteristics, quantified ammonia oxidation parameters, and *amoA* sequences were performed with the Paleontological Statistics (PAST) software package (Hammer et al. 2001) version 4.03. For each group of data to be compared, outliers were removed with the help of box plots. Relationships between numbers of *amoA* gene copies and abiotic soil parameters were established by non-parametric Spearman's rank order correlation analyses. Medians of univariate groups were compared by the non-parametric Kruskal–Wallis test and significant differences between the medians were determined by Dunn's post hoc test. Ordinations of communities of aerobic

ammonia-oxidizing microorganisms were performed by multivariate Principal Coordinate Analysis (PCoA). Differences between two or more microbial communities were established by the non-parametric PERMANOVA (PERMutational ANalysis Of VAriance) test. For establishing temporal differences per habitat, sample groups were formed that were defined by sampling period, *i.e.*, before and after the start of RIM in the RIM-impoundment, and by impoundment, *i.e.*, RIM or non-managed. In this way, the effects of RIM could be determined independently of external factors, that influenced both impoundments.

Results

Thaumarchaeal and betaproteobacterial *amoA* gene copy numbers

Thaumarchaeal and betaproteobacterial *amoA* gene copy numbers were highly variable for the two impoundments, the habitats within impoundments and sampling periods, resulting in large coefficients of variation, *i.e.*, 145 and 122% on average for thaumarchaeal and betaproteobacterial *amoA* gene copy

numbers, respectively. When comparing impoundments and habitats separately over all years, the copy numbers of the thaumarchaeal *amoA* gene did not significantly differ between impoundments or habitats (Table 1). When zooming in on each habitat separately, the thaumarchaeal *amoA* copy numbers differed significantly between the impoundments at the start of the study with higher copy numbers in the dense habitat in the RIM impoundment (Table 1). The thaumarchaeal *amoA* copy numbers increased in time in both impoundments, but the increase was only significant for the dwarf and sparse habitats in the non-managed impoundment (Table 1, Supplementary Fig. 1). During the 2013–2014 period, differences in thaumarchaeal *amoA* copy numbers between the impoundments were not registered.

When impoundments and habitats were compared separately over years, the bacterial *amoA* copy numbers were significantly higher in the RIM impoundment before the implementation of RIM, and in the dense habitat compared to the dwarf habitat (Table 1). The bacterial *amoA* copy numbers increased over the years only in the dwarf habitat in the RIM impoundment. In the period 2013–2014, bacterial *amoA* copy numbers were significantly higher in the dense habitat

Table 1 Median log numbers of ammonia-oxidizing Thaumarchaea and Betaproteobacteria (AOA and AOB, respectively) per g of dry soil

Level of comparison	Sampling periods	Subgroups	Log AOA	Log AOB	Log (AOA/AOB)
Impoundment	2008–2014	Non-managed	3.73 a	2.73 a	0.99 a
		RIM	3.99 a	3.49 b	0.53 a
Habitat	2008–2014	Dwarf	4.01 a	2.68 a	1.15 b
		Sparse	3.80 a	2.83 ab	1.06 ab
		Dense	3.78 a	3.61 b	0.43 a
Dwarf habitat	2008–2009	Non-managed	3.34 a	2.44 a	1.06 a
	2013–2014	Non-managed	4.30 b	2.88 ab	1.51 a
	2008–2009	RIM	3.96 ab	2.51 a	1.11 a
	2013–2014	RIM	4.14 ab	3.33 b	0.21 a
Sparse habitat	2008–2009	Non-managed	3.55 a	2.70 a	0.40 a
	2013–2014	Non-managed	4.28 b	2.82 a	1.21 a
	2008–2009	RIM	3.57 ab	3.09 a	1.05 a
	2013–2014	RIM	4.01 ab	3.05 a	0.55 a
Dense habitat	2008–2009	Non-managed	2.78 a	2.49 a	0.39 a
	2013–2014	Non-managed	3.62 ab	2.96 ab	0.78 a
	2008–2009	RIM	4.21 b	3.95 bc	0.17 a
	2013–2014	RIM	4.96 b	5.73 c	-0.77 a

Differences in characters indicate significant differences between subgroups within level of comparison ($p < 0.05$, Dunn's post hoc test)

in the RIM impoundment (Table 1, Supplementary Fig. 2). The ratio between thaumarchaeal and betaproteobacterial *amoA* copy numbers did not differ significantly between the impoundments before the start of RIM, but the ratios were significantly higher in the dwarf habitat when compared to the dense habitat (Table 1). Comparing habitats separately, there were no significant differences between impoundments and between sampling periods. The ratios were generally larger than zero (log-transformed numbers), meaning that thaumarchaeal *amoA* copies outnumbered the bacterial gene copies in most of the samples. The only exception was observed in the group of samples collected in the dense habitat in the RIM impoundment after the start of RIM (Table 1).

The log value of the copy numbers of thaumarchaeal *amoA* genes were significantly and positively correlated with the log value of the copy numbers of betaproteobacterial *amoA* genes (Table 2). Both log values were significantly and negatively correlated with Principal Component 1 (PC1) that originates from a Principal Component Analysis based on measured physicochemical soil properties (Laanbroek et al. 2024). In the original PCA analysis, PC1 was mainly determined by salinity-related properties. The ratio between the log values of AOA and AOB was significantly and negatively correlated with Principal Component 2 (PC2), which is largely determined by soil moisture content and by total and labile organic nitrogen. Remarkably, the potential nitrifying activity

(PNA) was significantly and positively correlated with the ratio between the numbers of thaumarchaeal and betaproteobacterial *amoA* gene copies.

Composition thaumarchaeal ammonia-oxidizing communities

The study yielded a total of 2,483,503 sequences of the partial thaumarchaeal *amoA* gene from 120 soil samples collected. Ten samples that had less than 8,000 sequences were excluded from further analysis. The data were initially rarefied to the size of the smallest sample (8,370). A total of 327 amplicon sequence variants (ASVs) were obtained. Of these 327 ASVs, nine were assigned to Bacteria, and three could not be assigned to Archaea or Bacteria. Of the 310 thaumarchaeal ASVs, 200 were tentatively identified as belonging to the order Nitrosopumilales, 87 to the order Nitrososphaerales, and 23 to the order Nitrosotaleales. The 23 most abundant ASVs with individual shares of at least 1% in the total number of sequences all belonged to the order Nitrosopumilales. The 23 most abundant ASVs formed the largest part of the ASV assemblies in the dwarf and sparse habitats in both impoundments, but not in the dense habitats, although their total share in the assemblies increased after the onset of intentional summer flooding (Fig. 1). Sequences classified as Nitrososphaerales and Nitrosotaleales were generally more abundant in samples collected from the dense habitat

Table 2 Spearman rank order correlations between the log of the numbers of thaumarchaeal and betaproteobacterial *amoA* gene copies (AOA and AOB, respectively), their ratio, the potential nitrification activity (PNA) and the first two Princi-

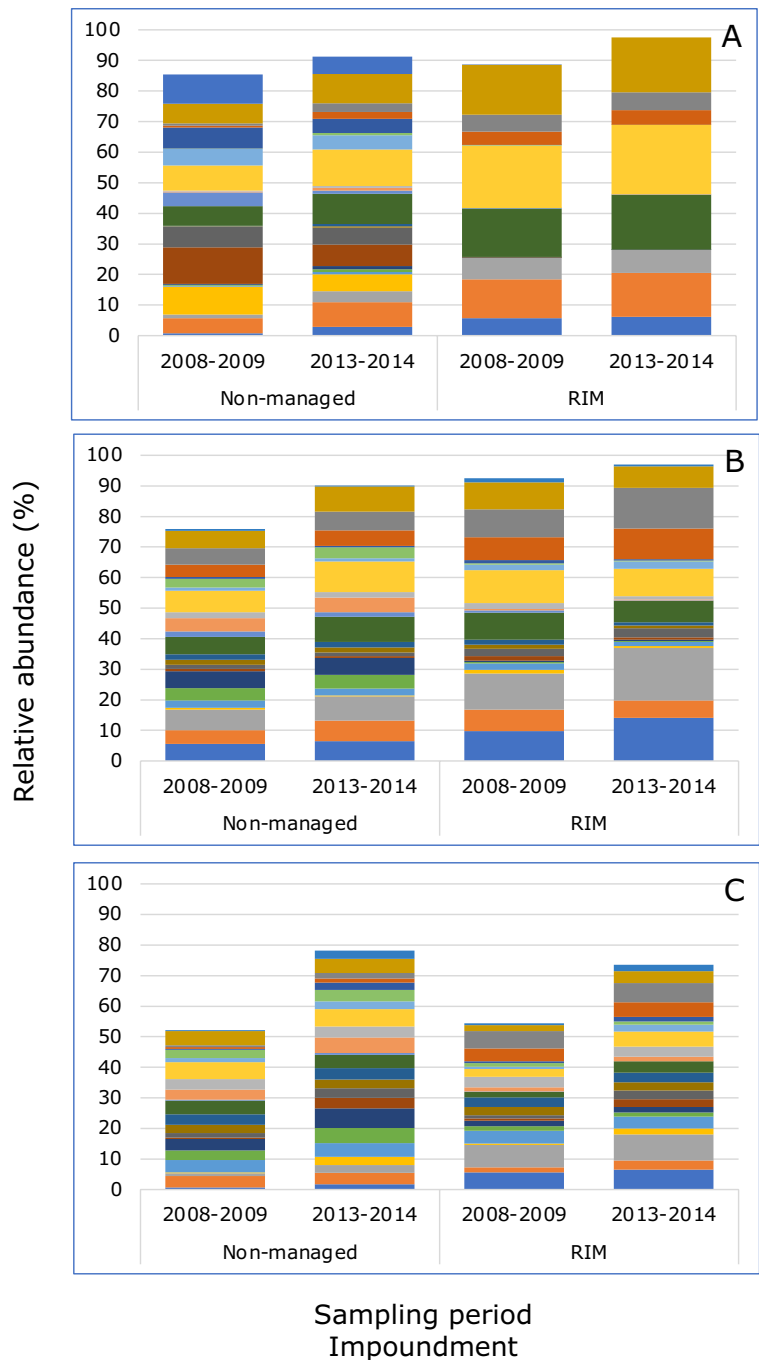
pal Components 1 and 2 (PC1 and PC2) of a Principal Component Analysis based on soil characteristics measured in March 2008, 2009, 2013 and 2014

	Log AOA	Log AOB	Log (AOA/AOB)	PNA	PC1	PC2
Log AOA		0.0039	0.0000	0.0710	0.0146	0.2394
Log AOB	0.2660		0.0000	0.0859	0.0160	0.1685
Log (AOA/AOB)	0.5292	-0.5722		0.0141	0.9672	0.0003
PNA	0.4489	0.3591	0.8808		0.1075	0.0449
PC1	-0.2263	-0.2232	0.0039	-0.1477		0.8754
PC2	0.1101	0.3880	-0.3335	0.1835	0.0145	

PC1 and PC2 explain 30.6 and 20.1%, respectively, of the total variance in abiotic parameters among the soil samples. PC1 is mainly determined by salinity-related characteristics (positively), while PC2 is largely determined by moisture, and total and labile organic nitrogen (all positively), and by dry bulk density and pH (both negatively) (Laanbroek et al. 2024)

p values are presented above the diagonal and Spearman's rho values below the diagonal

Fig. 1 The effect of space and time on the distribution of the most abundant ASVs of ammonia-oxidizing Thaumarchaea in the dwarf (A), sparse (B), and dense (C) habitats in the non-managed and in the RIM impoundment in the periods 2008–2009 and 2013–2014. The most abundant ASVs are defined as ASVs with an overall distribution of at least 1% of the total number of amplicons. Each ASV has corresponding colors in each of the columns



(Supplementary Fig. 3) and the numbers declined after the start of RIM, while sequences classified as Nitrosotaleales were even not detected anymore after the start of RIM.

Impoundment, habitat, and sample group as defined by impoundment and time of sample

collection each had a significant effect on the assemblies of thaumarchaeal *amoA* ASVs (Table 3). The exceptions were the temporal comparisons for each of the samples groups, *i.e.*, between sample group 1 and sample group 2, and between sample group 3 and sample group 4 in Table 3. When zooming in again

Table 3 Results of permutational multivariate analyses of variance (PERMANOVA) and PERMANOVA pairwise comparisons at the ASV taxonomic level of the ammonia-oxidizing thaumarchaeal and betaproteobacterial *amoA* communities

Analysis	Source	Thaumarchaea		Betaproteobacteria	
		F	p	F	p
One-way PERMANOVA	Impoundment	8.35	0.0001	7.65	0.0001
Pairwise comparison impoundments	Non-managed x RIM		0.0002		0.0001
One-way PERMANOVA	Habitat	11.59	0.0001	6.91	0.0001
Pairwise comparisons habitats	Dwarf x sparse		0.0001		0.0001
	Dwarf x dense		0.0001		0.0001
	Sparse x dense		0.0001		0.6862
	Sample groups ¹	3.68	0.0003	5.59	0.0001
Pairwise comparisons sample groups	Group 1 × group 2		0.1111		0.0196
	Group 1 × group 3		0.0006		0.0029
	Group 1 × group 4		0.0003		0.0001
	Group 2 × group 3		0.0009		0.2525
	Group 2 × group 4		0.0049		0.0012
	Group 3 × group 4		0.5887		0.0003

¹ Sample group 1: non-managed, 2008–2009; sample group 2: non-managed, 2013–2014; sample group 3: RIM, 2008–2009; sample group 4: RIM, 2013–2014

Two different factors were analyzed as possible drivers of community structure, *i.e.*, impoundment (non-managed and RIM) and habitat (dwarf, sparse and dense). Differences in community structure were assessed using Bray–Curtis dissimilarity

on each habitat separately, no significant temporal changes in ASV assemblies were observed in none of the habitats (Table 4). In contrast, ASV assemblies were significantly different between the non-managed and the RIM impoundment before and after the onset of intentional summer flooding in the dwarf and sparse habitats, but not in the dense habitat. The distribution of thaumarchaeal subclades present in the

23 most abundant ASVs differed over the habitats. (Supplementary Fig. 4).

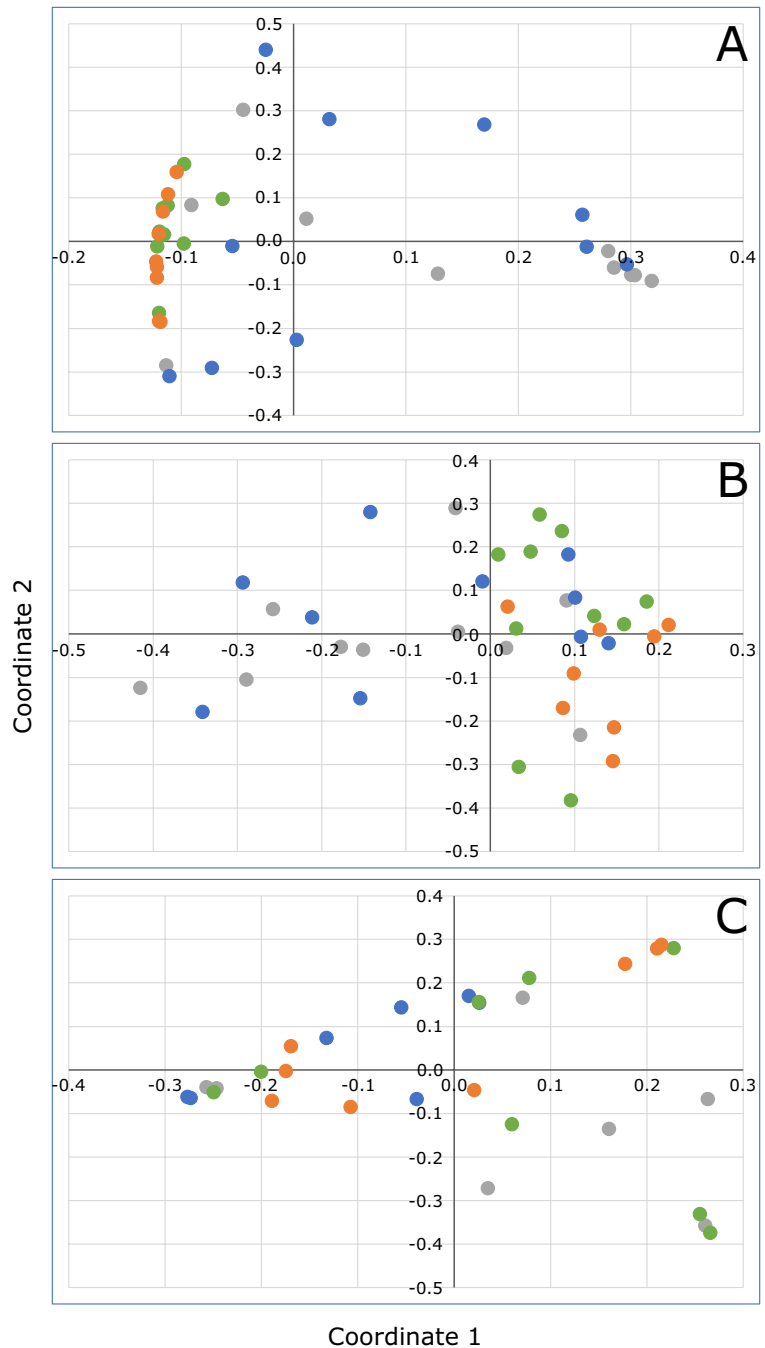
The distribution of ASVs in the dwarf and sparse habitats of the non-managed impoundment was more affected by the first two Principal Coordinates than the ASVs in the same habitats of the RIM impoundment (Fig. 2A, B). In the dwarf habitat, there were no differences in the two

Table 4 Results of permutational multivariate analyses of variance (PERMANOVA) pairwise comparisons between sample groups at the ASV taxonomic level of the ammonia-oxidizing thaumarchaeal and betaproteobacterial *amoA* communities

Domain	Pairwise comparisons	Habitat		
		Dwarf	Sparse	Dense
Archaea	(non-managed, 2008–2009) x (non-managed, 2013–2014)	0.3817	0.2654	0.0856
	(non-managed, 2008–2009) x (RIM, 2008–2009)	0.0002	0.0018	0.4547
	(non-managed, 2013–2014) x (RIM, 2013–2014)	0.0020	0.0026	0.3155
	(RIM, 2008–2009) x (RIM, 2013–2014)	0.1949	0.1275	0.7556
Bacteria	(non-managed, 2008–2009) x (non-managed, 2013–2014)	0.1753	0.0087	0.6927
	(non-managed, 2008–2009) x (RIM, 2008–2009)	0.0012	0.0053	0.9942
	(non-managed, 2013–2014) x (RIM, 2013–2014)	0.2008	0.0158	0.1831
	(RIM, 2008–2009) x (RIM, 2013–2014)	0.0004	0.0501	0.0769

Sample groups have been based on impoundment, *i.e.*, non-managed or RIM, in combination with sampling period, *i.e.*, before (2008–2009) and after (2013–2014) the onset of intentional summer flooding

Fig. 2 The ordination of assemblies of thaumarchaeal ASVs along the first two principal coordinates emerging from a Principal Component Analysis of soil samples collected from the dwarf (A), sparse (B) and dense (C) habitats in the non-managed impoundment in the periods 2008–2009 (grey symbols) and 2013–2014 (blue symbols), and in the RIM impoundment in the periods 2008–2009 (green symbols) and 2013–2014 (orange symbols). PC1 represents 62.4 (dwarf), 33.4 (sparse), and 26.7% (dense), respectively, of the variances between the assemblies, and PC2 11.0 (dwarf), 16.4 (sparse), and 23.5% (dense), respectively



impoundments. In the sparse habitat, the distribution of ASVs in the RIM impoundment, but not in the non-managed impoundment, shifted along the Y-axes of the PCoA plot after the implementation of intentional summer flooding. In the dense habitat, the distribution of ASVs along the first

two Principal Coordinates was comparable for both impoundments, and no effect of RIM was observed (Fig. 2C). The first two Principal Coordinates explained 73.2, 49.9, and 50.2% of the total variance between the assemblies in the dwarf, the sparse, and the dense habitats, respectively.

Composition betaproteobacterial ammonia-oxidizing communities

Of the 120 soil samples collected in March 2008, 2009, 2013, and 2014, 95 yielded fragments of the bacterial *amoA* gene. The data were rarefied to the size of the smallest sample (4,818) which resulted in 362 different ASVs. A total of 13 ASVs could not be assigned to Bacteria. No thaumarchaeal *amoA* gene fragments were detected nor were any *amoA* gene fragments of Nitrospirota (comammox bacteria) identified. Of the 349 bacterial ASVs, 337 were classified as Betaproteobacteria. Of these betaproteobacterial ASVs, 277 were classified as belonging to the family Nitrosomonadaceae. Of this family 170 ASV were further classified as uncultured *Nitrosomonas*, nine as *Nitrosomonas marina*, two as *Nitrosomonas eutropha* and seven as *Nitrosomonas* sp., and 17 as uncultured *Nitrosospora*. The 19 most abundant ASVs formed the largest part of the ASV assemblies in all habitats, especially so in the sparse habitat (Fig. 3).

Impoundment, habitat, and sample groups as defined by impoundment and time of sample collection had a significant effect on the assemblies of bacterial *amoA* ASVs (Table 3). When zooming in again on each habitat separately, significant temporal changes in ASV assemblies were observed in the sparse habitat of the non-managed impoundment and the dwarf habitat of the RIM impoundment, while nearly significant temporal changes were observed in the sparse and dense habitats of the latter impoundment (Table 4). There were significant spatial differences between corresponding habitats in both impoundments in the dwarf and sparse habitats before the onset of intentional summer flooding and in the sparse habitat after the start of RIM.

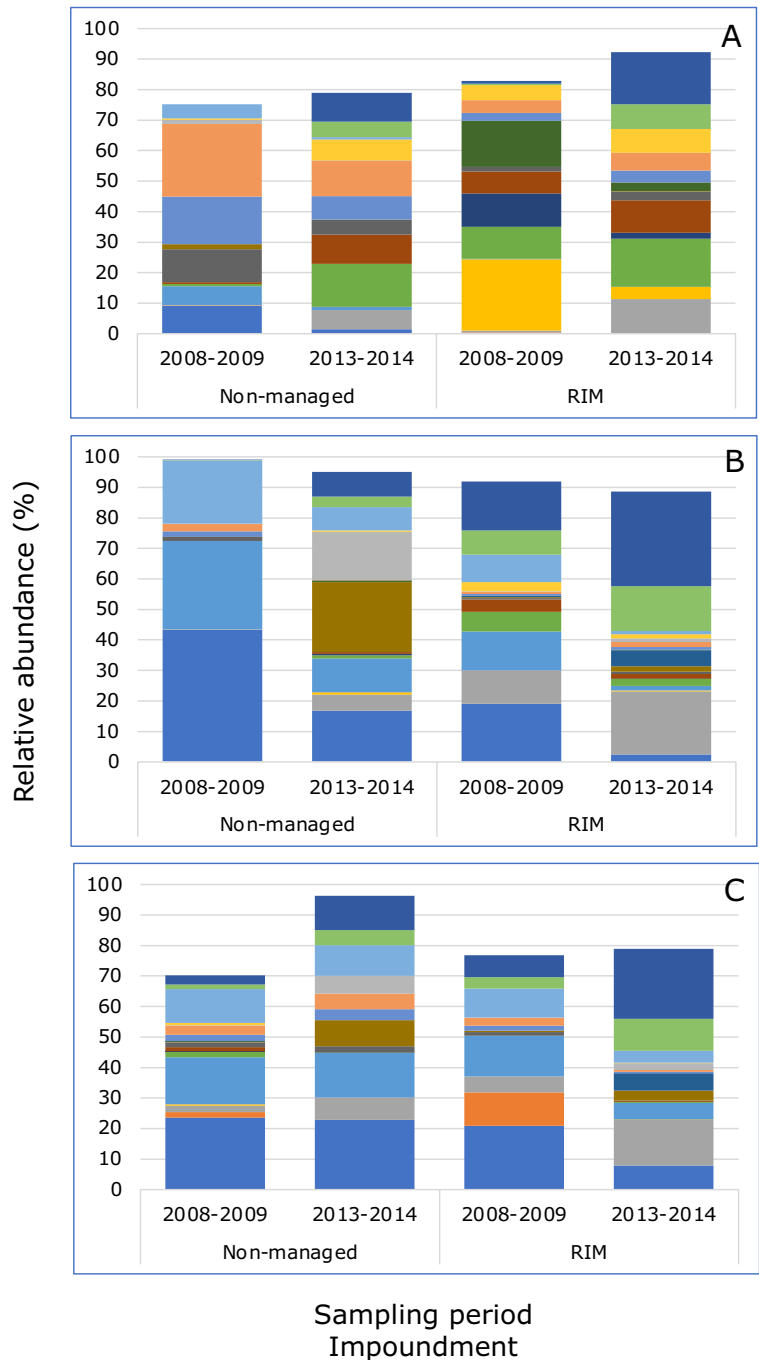
Most assemblies of *amoA*-based ASVs in the non-managed impoundment are separated from most assemblies in the RIM impoundment, and the samples collected in the latter impoundment in the period 2008–2009 cluster separately from the samples collected in this impoundment in the period 2013–2014 (Fig. 4A). The spatial and temporal separation of samples observed in the dwarf habitat were not visible in the sparse and dense habitats of either impoundment, although in the RIM impoundment most assemblies detected in the period 2013–2014 seem to differ spatially from most assemblies found in the period 2008–2009 (Fig. 4B, C). The first two Principal

Coordinates explained 45.3, 74.3, and 57.6% of the total variance between the assemblies in the dwarf, the sparse, and the dense habitats, respectively.

Discussion

While a temporal decrease in potential nitrifying activities were found in the dwarf and sparse habitats of both impoundments after the implementation of RIM, although only significantly in the dwarf habitat of the non-managed impoundment (Laanbroek et al. 2024), the numbers of thaumarchaeal *amoA* copies increased significantly in the dwarf and sparse habitats in the non-managed impoundment, but not in the RIM impoundment. The effect of RIM on thaumarchaeal *amoA* gene copy numbers were found only at the higher parts of the non-managed impoundment that were not directly influenced by inundation. Hence, unknown external factors must have been responsible for the effect on thaumarchaeal *amoA* gene copy numbers in the dwarf and sparse habitats in the non-managed impoundment. Intentional summer flooding may have counteracted such external factors in the RIM impoundment leading to the absence of a temporal response at the higher elevations in that impoundment. Hence, this may be an indirect effect of intentional summer flooding. Numbers of betaproteobacterial *amoA* copies changed only significantly in time in the dwarf habitat in the RIM impoundment. Only the dwarf habitat had a significant increase in moisture content and significant decrease in salinity due to RIM, which may have stimulated growth of ammonia-oxidizing Betaproteobacteria. The nitrogen-use efficiencies in mangroves are among the highest recorded for angiosperms (Reef et al. 2010) and ammonium was depleted in the root zone of the mangrove species *Kandelia obovata* (Alongi et al. 2005). Hence, growth of ammonia-oxidizing Betaproteobacteria at the higher elevations of the RIM impoundment simultaneously with the expansion of mangrove forest suggests was likely not ammonium limited. Moreover, when ammonium limitation due to vigorous plant growth would have been the case, Thaumarchaea with their higher affinities for ammonium compared to the ammonia-oxidizing Betaproteobacteria would have come to the fore and not the ammonia-oxidizing Betaproteobacteria (Martens-Habbena et al. 2009; Park et al. 2010; Jung et al. 2011, 2021; Kits

Fig. 3 The effect of space and time on the distribution of the most abundant ASVs of ammonia-oxidizing Betaproteobacteria in the dwarf (A), sparse (B), and dense (C) habitats in the non-managed and in the RIM impoundment in the periods 2008–2009 and 2013–2014. The most abundant ASVs are defined as ASVs with an overall distribution of at least 1% of the total number of amplicons. Each ASV has corresponding colors in each of the columns

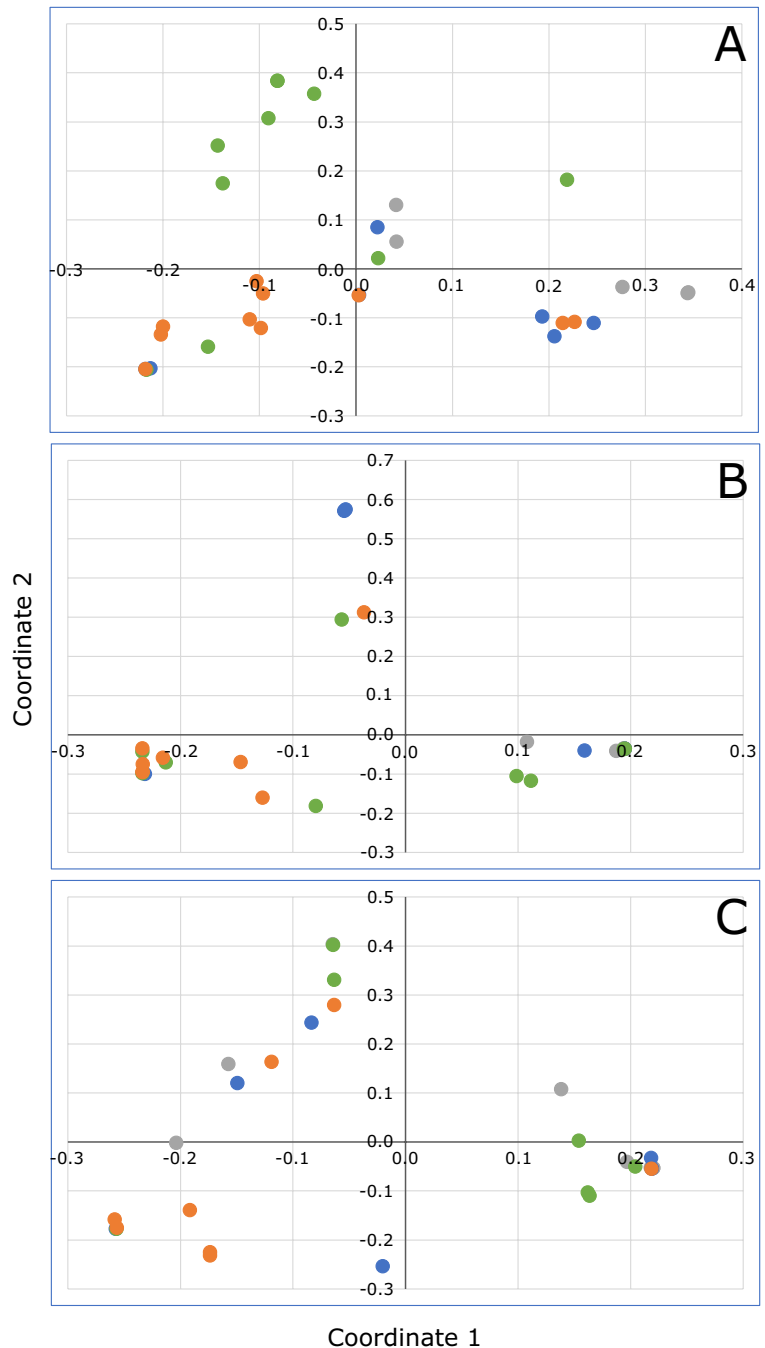


et al. 2017). Hence, some unknown soil factor must have stimulated the growth of the latter specifically in the dwarf habitat of the RIM impoundment.

In contrast to the numbers of ammonia-oxidizing thaumarchaeal and betaproteobacterial *amoA* gene numbers, the ratio between thaumarchaeal and

betaproteobacterial *amoA* copy numbers, correlated significantly with the second PC of a Principal Component analysis based on measured soil properties (Laanbroek et al. 2024). Soil moisture content, and total and labile organic nitrogen contributed mostly and positively to this second PC, and pH negatively.

Fig. 4 The ordination of assemblies of betaproteobacterial ASVs along the first two principal coordinates emerging from a Principal Component Analysis of soil samples collected from the dwarf (**A**), sparse (**B**) and dense (**C**) habitats in the non-managed impoundment in the periods 2008–2009 (grey symbols) and 2013–2014 (blue symbols), and in the RIM impoundment in the periods 2008–2009 (green symbols) and 2013–2014 (orange symbols). PC1 represents 24.7 (dwarf), 57.3 (sparse), and 40.4% (dense), respectively, of the variances between the assemblies, and PC2 20.7 (dwarf), 17.0 (sparse), and 17.2% (dense), respectively



Hence, moisture, total and labile organic nitrogen may be responsible to the promotion of Thaumarchaea over their betaproteobacterial counterparts, while pH may have favored ammonia-oxidizing Betaproteobacteria over their archaeal equivalents. Since soil moisture content could be considered as proxy of

oxygen availability, Thaumarchaea and Betaproteobacteria may be differently affected by oxygen availability, *i.e.*, the first group by high moisture contents and low oxygen availability, and the second by low moisture contents and high oxygen availability. The ratio between thaumarchaeal and betaproteobacterial

amoA copy numbers was larger than 10 in the dwarf habitat in the non-managed impoundment before and after the implementation of RIM, in the dwarf and sparse habitats in the RIM impoundment prior to the start of RIM, and in the sparse habitat in the non-managed impoundment after the onset of RIM. From culture experiments with ammonia-oxidizing Thaumarchaea and Betaproteobacteria from freshwater environments, French and colleagues concluded that for an equal contribution of both groups to ammonia oxidation, the ratio in abundance between the groups should be at least 10 to 100 times in favor of archaea (French et al. 2021). Based on the findings of French et al., the contribution of Thaumarchaea should be of limited importance in the dense habitat in both impoundments, and in the dwarf and sparse habitats in the RIM impoundment after the implementation of RIM. The ratio between thaumarchaeal and bacterial *amoA* copy numbers in the surface layer of a sediment close to *K. obovata* mangrove trees amounted to 0.20 (Li et al. 2011). In the sediment of another *K. obovata* mangrove forest this ratio was 0.30 (Wang et al. 2015). Hence, the ratios observed in the *K. obovata* mangrove forests suggest an even larger role of bacteria in the oxidation of ammonia than in the impounded *A. germinans* forest soils.

Ammonia-oxidizing Thaumarchaea and Betaproteobacteria may respond differently to changes in the physicochemical soil properties caused by RIM due to intrinsic differences in physiological properties. For example, thaumarchaeal and betaproteobacterial *amoA* copy numbers showed a different response with respect to salinity in a laboratory incubation experiment with sediment from a *K. obovata* mangrove forest (Wang et al. 2018). In contrast to the thaumarchaeal *amoA* gene, the bacterial gene showed higher numbers at intermediary salinity values (10 or 20 PSU) compared to low (0 PSU) or high (30 PSU) salinity values. A meta-analysis revealed that ammonia-oxidizing bacteria respond more strongly to nitrogen additions than ammonia-oxidizing archaea (Carey et al. 2016). Thaumarchaea have apparent affinities for ammonia and oxygen, which are substantially higher than observed for ammonia-oxidizing Betaproteobacteria (Martens-Habben et al. 2009; Park et al. 2010; Jung et al. 2011, 2021; Kits et al. 2017). This will render Thaumarchaea an advantage under ammonium- or oxygen-limited conditions. Hence, a change in salinity, ammonium and oxygen availability should

become visible in the ratios between thaumarchaeal and bacterial *amoA* gene copy numbers, unless changes with opposing effects on the ratio occur simultaneously, such as a decrease in salinity and in oxygen.

Notwithstanding the lack of a net increase in copy numbers of thaumarchaeal and betaproteobacterial *amoA* genes in most habitats, changes in the assemblies of communities based on amplicon sequences of these genes were observed. Due to the short sequence lengths used in the Illumina MiSeq sequencing it is not possible to classify the ASVs at the level of cultivated microbial genera or species, which makes it difficult to ascribe physiological characteristics to the ASVs and to relate these subsequently to observed changes in physicochemical soil conditions. Some of the most abundant thaumarchaeal ASVs were provisionally classified as Nitrosopumilales subclades NP- α -2, NP- γ -2, and NP- δ -1. The distribution of the subclades differed over the different sample groups and over the habitats. Subclade NP- α -2 was relatively most present in the sparse and dense habitats of the non-managed impoundment, and in the dense habitat of the RIM-impoundment (Supplementary Fig. 4). No effect of RIM was observed on the relative distribution of this subclade. Subclade NP- γ -2 was relatively most present in the dwarf and sparse habitats of both impoundments, and almost independent on the period of sampling. This subclade was most often found in the RIM impoundment. Subclade NP- δ -1 was mostly present in the dense habitats of both impoundments (Supplementary Fig. 4). Intentional summer flooding hardly affected the relative distribution of this subclade. Comparing the relative distribution of the subclades over the three habitats and the associated elevations, the contribution of subclades NP- α -2 and NP- δ -1 decreased with decreasing elevations at the cost of mostly non-annotated subclades in the non-managed impoundment. In the RIM impoundment, the relative contribution of subclade NP- γ -2, which was high in the dwarf habitat, diminished in favor of the other subclades and the non-annotated Nitrosopumilales subclades (Supplementary Fig. 4). The replacement of subclades along the elevation gradient is most likely due to changing conditions because of differences in elevation, *i.e.*, moisture content, and ammonium availability.

However, the main conclusion with respect to the most abundant Nitrosopumilales subclades is the

near absence of a clear effect of RIM on their relative distribution. Nitrosopumilales subclade NP- α -2 is mostly detected in marine waters and is not represented in enriched or pure cultures (Alves et al. 2018). Nitrosopumilales subclade NP- γ -2 is mostly revealed in marine and estuarine sediments (Alves et al. 2018). The species *Nitrosopumilus maritimus*, *Nitrosopumilus ureiphilus*, *Nitrosopumilus cobalamingenesis*, and *Nitrosopumilus oxycliniae*, and several enrichment cultures, e.g., *Ca. Nitrosopumilus salarius*, *Ca. Nitrosopumilus piranensis*, *Ca. Nitrosopumilus sediminis*, *Ca. Nitrosopumilus koreense*, and *Ca. Nitrosopumilus adriaticus* belong to this subclade. Members of the genus *Nitrosopumilus* have all an aerobic, chemolithotrophic way of life, using the conversion of ammonia to nitrite as energy source for the assimilation of CO₂ to organic molecules (Qin et al. 2017). Their apparent half-saturation constants (K_m) for O₂ uptake and ammonia oxidation are relatively low (Martens-Habbenha et al. 2009), which give the *Nitrosopumilus* species a competitive advantage in water-saturated, oxygen- and ammonium-limited mangrove forest soils. Nitrosopumilales subclade NP- δ -1 is mostly detected in marine and estuarine sediments and is not represented in enriched or pure cultures (Alves et al. 2018). ASVs provisionally classified as Nitrososphaerales or Nitrosotaleales were less abundant than the ASVs classified as Nitrosopumilales and disappeared almost or completely after the onset of RIM. The ASVs classified as Nitrososphaerales or Nitrosotaleales belonged to the subclade NS- β -1 and NT- α -1, respectively (Alves et al. 2018). Representatives of the subclade NS- β -1 and NT- α -1 are predominantly found in terrestrial soils and sediments, but sometimes also in marine sediments. Subclade NS- β -1 does not contain pure or enriched species, but subclade NT- α -1 contains the enriched *Ca. Nitrosotalea devanattera* and *Ca. Nitrosotalea* sp. Nd2 species (Alves et al. 2018). Both enriched *Ca. Nitrosotalea* species are obligate acidophilic (Prosser and Nicol 2015) and therefore most likely not active under the prevailing pH neutral conditions present in mangrove forest soils.

Since 2015, the range of aerobic ammonia-oxidizing microorganisms has been extended with comammox *Nitrospira* species (Daims et al. 2015; Van Kessel et al. 2015). In contrast to the aerobic ammonia-oxidizing Thaumarchaea and Betaproteobacteria that both oxidize ammonia to nitrite, which

is then further oxidized to nitrate by aerobic nitrite-oxidizing bacteria, comammox *Nitrospira* species oxidize ammonia directly to nitrate. Comammox *Nitrospira* species have a high affinity for ammonia, which is three orders of magnitude greater than the affinities of ammonia-oxidizing Betaproteobacteria and non-marine Thaumarchaea for ammonium and are therefore supposed to be active under oligotrophic conditions (Sakoula et al. 2021). Comammox amoA genes have been found in many habitats, including in six mangrove forest soils across southeastern China (Liu et al. 2020; Meng et al. 2022). We were not able to detect copies of comammox amoA genes in the impoundments along the Indian River Lagoon. In contrast to us, Liu and colleagues (Liu et al. 2020) used a nested PCR protocol for the amplification of comammox amoA genes used for high-throughput sequencing. The authors suggested that the nested protocol may have been the reason for the high comammox amoA diversity observed in their soils. When looking at amoA copy numbers by quantitative PCR, Liu and colleagues observed slightly more comammox amoA copies than betaproteobacterial amoA copies, but both were significantly lower than thaumarchaeotal amoA copies. Recently, it has been demonstrated that not all primer sets developed for the detection of comammox *Nitrospira* species showed the presence of these ammonia-oxidizing bacteria in mangrove soils (Meng et al. 2022). Some of the primer sets detected comammox gene copies at the same level as gene copies of the ammonia-oxidizing Proteobacteria. Hence, comammox bacteria might also have been present in the soils of the impounded mangrove forests but remain undetected by the primer set applied by us.

Final remarks

The short sequence lengths of the amoA amplicons used for MiSeq sequencing make it impossible to link changes in ASVs to changes in environmental conditions that are caused by adaptations in the management of impounded mangrove forests. For such a link it is necessary to have knowledge of ASVs at the level of genera and preferably even species. In a former study (Laanbroek et al. 2012), which involved only the first year after the onset of RIM, we found a relative increase in the shares of *Nitrosomonas* sp. NM143 and *Nitrosospira* cluster 1

at the expense of the share of *Nitrosomonas marina* in the total ammonia-oxidizing bacterial community. This former study had been based on the constructions of bacterial 16S rRNA clone libraries with longer sequence lengths, which yielded identifications at the level of species.

Differences in hydrology between the two impoundments had led to differences in the communities of aerobic ammonia-oxidizing microorganisms already at the start of our study. Before the start of RIM, numbers of Thaumarchaea in the dwarf and sparse habitats were significantly larger in the RIM impoundment. In the dwarf and sparse habitats, the composition of the aerobic ammonia-oxidizing communities of Thaumarchaea and of Betaproteobacteria were significantly different between both impoundments. So, it may be questioned whether the non-managed impoundment has been a suitable control for the management intervention in the RIM impoundment as the aerobic ammonia-oxidizing microbial communities have been different from the start of the study. The suitability of the adjacent, non-managed impoundment as a control for the RIM impoundment has been questioned before in the parallel study (Laanbroek et al. 2024) as RIM affects the non-managed impoundment by an exchange of groundwater between both impoundments. Nevertheless, the current study has shown the effects of intentional summer flooding on the aerobic ammonia-oxidizing communities in impounded mangrove forests, especially so at higher elevations, which could also tell us about the effects of climate-driven sea level rise on the diversity of aerobic ammonia-oxidizing microorganisms in mangrove ecosystems.

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Author contributions HJL collected the field samples, MMH isolated the DNA and performed the quantitative gene measurements, OYAC executed the sequencing data processing and amplicon identification, HJL analyzed the data and wrote the manuscript draft, MMH, OYAC and EEK contributed critically to the manuscript drafts, and gave final approval for publication.

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Data availability All data used are presented in the Supplementary Information.

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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